Testicular development of Zebu bulls after chronic treatment with a gonadotropin-releasing hormone agonist[1]

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ABSTRACT: The objective was to compare testis characteristics of Zebu bulls treated with the GnRH agonist, deslorelin, at different times and for different durations during their development. An additional objective was to determine the usefulness of a stain for the transcription factor GATA-binding protein 4 (GATA-4) as a specific marker for Sertoli cell nuclei in cattle. Bulls (54) were allocated to nine groups (n = 6) and received s.c. deslorelin implants as follows: G1 = from birth to 3 mo of age; G2 = from 3 to 6 mo; G3 = from 6 to 9 mo; G4 = from 9 to 12 mo; G5 = from birth to 15 mo; G6 = from 3 to 15 mo; G7 = from 6 to 15 mo; G8 = from 12 to 15 mo; and G9 (control) = no implant. Bulls were castrated at 19 mo of age. Paraffin sections (10 μm) were subjected to quantitative morphometry and GATA-4 immunohistochemistry. At castration, all bulls in the control group (6/6) had attained puberty (scrotal circumference ≥ 28 cm), whereas a smaller proportion ($P < 0.05$) had reached puberty in G2 (2/5) and G6 (1/6). Bulls in G2 and G6 also had a lesser ($P < 0.05$) testis weight compared with the control group. Total volume of seminiferous epithelium and total daily sperm production in G2 and G6 were only half that observed in the control group. Spermatids were observed in less than 50% of seminiferous tubules in G2, G6, and G7 compared with 82% in the control group ($P < 0.05$). Staining for GATA-4 was specific for and abundant in the Sertoli cell nucleus in both pre- and postpubertal bulls, and no other cell nucleus inside the seminiferous tubule was positive for GATA-4. Total number of Sertoli cells was not affected by treatment ($P = 0.45$), but nuclear volume was smaller in G2 and G6 ($P < 0.05$) compared with the control group. In conclusion, treatment of Zebu bulls with deslorelin had no apparent beneficial effect on testis development and delayed puberty when treatment was initiated at 3 mo of age. Staining for GATA-4 was a useful method for identifying and quantifying Sertoli cell nuclei in both pre- and postpubertal bulls.

Key Words: GATA-Binding Protein 4, Gonadotropin-Releasing Hormone, Male Sexual Function, Sertoli Cells, Testis Development, Zebu Cattle

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Introduction

Prepubertal and young postpubertal bulls treated with GnRH agonists have increased testosterone (**T**) concentration in blood and testes (Melson et al., 1986; Aspden et al., 1998), and apparent enhancement of steroidogenic and spermatogenic capacities (D'Occhio and Aspden, 1996; Aspden et al., 1998). Increased secretion of T in GnRH-treated bulls likely results from the increased basal concentration of LH (Jiménez-Severiano et al., 2003), increased numbers of LH receptors (Melson et al., 1986), or both. In contrast, testicular development is impaired and sperm production is decreased after puberty when GnRH agonist is administered to bull calves at relatively young ages (Chandolia et al., 1997). Furthermore, when treatment with GnRH ago-

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nist is discontinued in adult bulls, pituitary function is impaired as shown by decreased response to exogenous GnRH (Bergfeld et al., 1996) and lower amplitude of LH pulses (Jiménez-Severiano, 2001) for at least 20 to 40 d. It has been suggested that the increased intratesticular and plasma concentrations of T, induced by treatment with GnRH agonists, might be beneficial in situations where T production is decreased and is limiting sperm production and semen quality (D'Occhio and Aspden, 1999). This might be particularly important in hastening sexual development and improving testis function in less precocious animals, such as bulls from *Bos indicus* breeds, which reach puberty at older ages compared with *B. taurus* bulls (Chase et al., 1997; Lunstra and Cundiff, 2003). Given the apparent contrasting response to GnRH agonist treatment in animals of different ages, the present experiment was conducted to compare the development and function of testes and sperm production after puberty of *B. indicus* bulls treated with a GnRH agonist, starting at different times and for different durations of treatment during their development between birth and puberty.

Materials and Methods

Experimental Design and Treatments

All experimental procedures were reviewed and approved by the animal ethics committee at James Cook University (**JCU**). This experiment was conducted at the experimental station of JCU in northern Queensland (Fletcherview), located in the dry tropics of eastern Australia. Fifty-four crossbred bull calves (7/8 *B. indicus*, 1/8 *B. taurus*) were used. Bulls were maintained in large paddocks, where they had ad libitum access to native forage and water in the manner that cattle are typically managed in northern Queensland. Bulls were allocated randomly to nine groups (n = 6 per group). Three bulls died during the first months of the experiment, and one bull had atrophy of one testis; therefore, all data from these animals were discarded. Three more bulls died near the end of the experiment, and only data at castration and testis morphometry were not available for these bulls. None of the deaths of bulls were related to treatments applied. Bulls from each group were treated at varying ages, as described below, with a bioimplant containing 12 mg of the GnRH agonist deslorelin (Peptech Animal Health Pty. Ltd., Sidney, Australia); the characteristics of these implants have previously been described (D'Occhio et al., 1996). Implants were placed s.c. in the ear under aseptic conditions by means of a commercial device and replaced with a fresh 12-mg implant after 6 mo of use in those animals with more than 6 mo of treatment. The schedule of treatment in the different experimental groups was as follows: **G1** = from birth to 3 mo of age; **G2** = from 3 to 6 mo; **G3** = from 6 to 9 mo; **G4** = from 9 to 12 mo; **G5** = from birth to 15 mo; **G6** = from 3 to 15 mo; **G7** = from 6 to 15 mo; **G8** = from 12 to 15 mo; and **G9** (control) = received no deslorelin implant. In G5, G6, G7, and G8, implants were removed at 15 mo of age because at that age bulls in the control group had attained puberty, according to the established criteria (subsequently described).

Monthly Measurements and Blood Sampling

Scrotal circumference (**SC**) and BW were evaluated at monthly intervals from 2 to 18 mo of age. Bulls achieving a SC of 28 cm were considered to have attained puberty (Lunstra et al., 1978; Lunstra and Cundiff, 2003). The age and BW when this SC size was attained were recorded for each bull. Additionally, blood samples were collected every month to determine LH and T concentrations.

Sample Collection and Histological Preparation

At an average age of 19 mo, bulls were surgically castrated under local anesthesia. Testes and epididymides were trimmed of excessive connective tissue and weighed separately. The left testis from each bull was cut longitudinally and two samples of approximately 10 g of testicular parenchyma were recovered from each bull, immersed in Dulbecco's PBS (0.15 *M*, pH 7.4), and frozen at –20°C. These samples were used to determine daily sperm production (**DSP**) by counting the number of spermatids in homogenized tissue, according to a method previously described (Amann, 1970). A time divisor of 5.32 was used to estimate the DSP per gram of tissue and per paired testes (Amann et al., 1974; Lunstra and Cundiff, 2003).

Two additional 1-cm^3 samples of testicular parenchyma were collected from the same region of the left testis of each bull, and fixed in 4% paraformaldehyde in Dulbecco's PBS, with gentle agitation. After 4 h of fixation, fixative was replaced with freshly thawed solution and fixation was continued overnight with gentle agitation. Samples were processed until embedding in paraffin wax as previously described (McCoard et al., 2001), except that samples remained in 70% ethanol for approximately 4 mo before proceeding with the rest of the dehydration process. Five serial 10-μm sections were taken from each sample, dried overnight on glass slides at 37°C, and stored at room temperature until processing for immunohistochemistry as subsequently described.

Immunohistochemistry Procedures

Localization of GATA-Binding Protein 4. Tissue sections were subjected to immunohistochemistry for localization of the transcription factor GATA-binding protein 4 (**GATA-4**) as a marker for Sertoli cell nuclei, as previously described (McCoard et al., 2001). This transcription factor is abundant in Sertoli cell nuclei but not in germ cells in the seminiferous tubules during fetal and early postnatal life in mice (Viger et al., 1998) or during fetal, prepubertal, and adult life in pigs

(McCoard et al., 2001). An affinity-purified goat polyclonal antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:100 dilution. This antibody was developed against a peptide mapping at the carboxy terminus of GATA-4 of mouse origin. This epitope is highly conserved between mice and other mammals such as humans and pigs (at the time this manuscript was written, no sequence for bovine GATA-4 had been published). Localization of the antigen-antibody complex was achieved using the avidin-biotin-immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA), and Novared (Vector Laboratories Inc.) as the chromagen. Sections were lightly counterstained with hematoxylin, dehydrated, cleared, and mounted. Slides were stored at room temperature until morphometric analysis was conducted.

Validation of GATA-4 Staining as a Marker for Bovine Sertoli Cell Nuclei. Additional tissue sections taken from prepubertal and postpubertal bulls were subjected to the same immunohistochemistry procedures described above, but with a GATA-4 blocking peptide (Santa Cruz Biotechnology) 10× and 20× in excess of the primary antibody to confirm the specificity of the GATA-4 antibody. Furthermore, nonimmune serum or buffer solution with no primary antibody was used to confirm that nonspecific binding was not problematic. In addition, primary antibody was tested at different dilutions (1:100, 1:200, 1:400, 1:600, and 1:800) by either 1-h or overnight incubation at room temperature. The 1:100 by 1-h and 1:200 overnight incubations provided for optimal staining compared with the other dilution and time combinations. The identification of GATA-4 positive nuclei as Sertoli cell nuclei was made based on cell morphology and position of the nuclei in the seminiferous epithelium.

Stereological Evaluation of the Testicular Sections

The 10-μm sections stained for GATA-4 were observed under a bright-field microscope using computerized morphometry planimetry (Bioquant IV system, R & M Biometrics, Nashville, TN). Two tissue sections from each bull were used for these evaluations, and four areas representing each quadrant on each section were evaluated. The volume percentage of the testicular parenchyma composed of seminiferous tubules or interstitial tissue were determined at a magnification of 200×. Tubular components of the parenchyma were measured by tracing outlines of whole seminiferous tubules (approximately 120 tubules per bull) using a computer mouse connected to a high-resolution digital pad. For seminiferous epithelium and Sertoli cell nuclei evaluation, four round tubules were selected within each quadrant at 1,000× magnification. Seminiferous epithelium was measured by tracing the epithelium outline of half of each tubule. Sertoli cell nuclei were touch counted, and outlines also were traced to measure the perimeter of each nucleus (about 600 nuclei per bull were measured). The average volume of each nucleus was calculated using the formula for a prolate sphere, adjusted for capping effects using Abercrombie's formula, as described by McCoard et al. (2001). The total number of Sertoli cells per testis was determined using the formula of Wreford (1995), where the total number of Sertoli nuclei per testis was the product of testis volume and volume density of nuclei in the testis (i.e., total mass of Sertoli nuclei per testis) divided by the mean nuclear volume. As fixed samples exhibited a large amount of deformation and shrinkage, a correction for tubule shrinkage was applied based on the traced perimeter of each shrunken tubule divided by the traced perimeter of the footprint (curved edges of the interstitium) around each tubule. In our experience, this approach does not introduce any errors to these measurements or to subsequent calculations.

Hormone Radioimmunoassays

Concentrations of LH were determined by a double-antibody RIA (Wolfe et al., 1989). The limit of detection was 259 pg/mL, and the intra- and interassay CV were 3 and 12%, respectively. Concentrations of T were determined by a solid phase assay (Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA). The limit of detection was 28 pg/mL, and the intra- and interassay CV were 6 and 7%, respectively. Validation of the RIA for T was as follows: several tests for parallelism were performed using different samples (i.e., control standards from the kit, bull serum samples, serum from ovariectomized cows spiked with T and bovine follicular fluid [**bFF**]). Concentration of T in these samples ranged from 1.36 to 10.41 ng/mL. Samples were 1:1 (not diluted) or diluted 3:4, 2:4, and 1:4 in either T zero calibrator from the kit or sera of ovariectomized cows, and assayed in quadruplets. The serial dilutions in the different tests generated binding inhibition curves that paralleled the T standard curve. Recovery of mass was evaluated by adding 25 μL of standard B, C, D, or E (from the kit) to 25 μL of different bFF pools or by adding 25 μL of standard B or C to 25 μL of bovine serum samples. The contribution of 25 μL of standards B, C, D, and E was assumed to be 0.1, 0.5, 2, and 4 ng/mL, respectively. The average ± SD recovery from these samples was 105 ± 4.9% for bFF and 108 ± 6.3% for bovine serum.

Statistical Analyses

Data for SC, BW, LH concentrations, and T concentrations were analyzed as a completely randomized design (**CRD**), using the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC), with the statement repeated, the options sub = animal nested within treatment, and autoregressive (1) covariance structure within animal. Treatment, age, and treatment × age interaction were included in the model. Characteristics at puberty, variables at castration, and daily sperm production were analyzed as a CRD using the GLM procedure of SAS. Variables for quantitative morphometry were analyzed

as a three-level nested design using the MIXED procedure of SAS. The model included the fixed factor of treatment and the random effects of bull nested within treatment, cross section nested within bull, and quadrant nested within section. Dunnett's test was used to compare least squares means between each treated and control group. Additional preplanned contrasts were conducted to compare variables at puberty and at castration, and testis morphometry between the control group and the next group combinations: G1+G5; G2+G6; or G3+G7 (grouping was made based on the age of bulls at the beginning of treatment). The Pearson χ^2 test was used to compare proportions of bulls reaching puberty in treated and control groups.

Results

Body Weight and Testicular Development

In bulls from the control group, SC increased throughout the experiment (Figure 1), with two relatively static periods of growth corresponding to the winter months in Australia. The effects of treatment ($P < 0.001$), age ($P < 0.001$), and the interaction of treatment × age ($P < 0.01$) were significant. Between 2 and 7 mo of age, no differences in SC were detected among groups. Scrotal circumference was smaller in G2 (from 8 through 18 mo of age), G6 (from 9 through 18 mo of age), G7 (from 12 through 18 mo of age), and G8 (from 12 to 14 mo of age) compared with the control group ($P < 0.05$). No effect of treatment or the interaction of treatment × age on BW was detected (data not shown), and BW consistently increased from an overall average of 109 ± 3.7 kg by 2 mo of age through 367 ± 3.7 kg at the end of the study.

Characteristics at Puberty

In the control, G3, and G5 groups, all bulls had attained puberty by the end of the experiment (Table 1). Bulls in control group attained puberty between 11 and 15 mo of age. There was a smaller proportion of bulls attaining puberty ($P < 0.05$) in G2 (2/5; at 15 and 17 mo of age) and G6 (1/6; at 18 mo of age) during the time the experiment was conducted. Of those bulls attaining puberty, only those in G7, G2+G6, and G3+G7 were older at puberty ($P < 0.05$) than bulls in the control group. Bulls in G2+G6 and G3+G7 were heavier at puberty compared with the control group ($P < 0.05$).

Characteristics at Castration

At castration, bulls in G2, G6, and G7 had smaller testes compared with the control group (Table 2) as indicated by the smaller SC and paired testis and parenchymal weights in G2 and G6 ($P < 0.05$) and G2+G6 ($P < 0.001$) and smaller SC in G7 ($P = 0.10$) compared with the control group. The weight of the epididymides also was less for bulls in G6 ($P = 0.06$) and G7 ($P = 0.09$) and G2+G6 ($P < 0.01$) compared with bulls in the control group. No differences were detected for BW at castration when treatment groups were compared with the control group ($P = 0.73$).

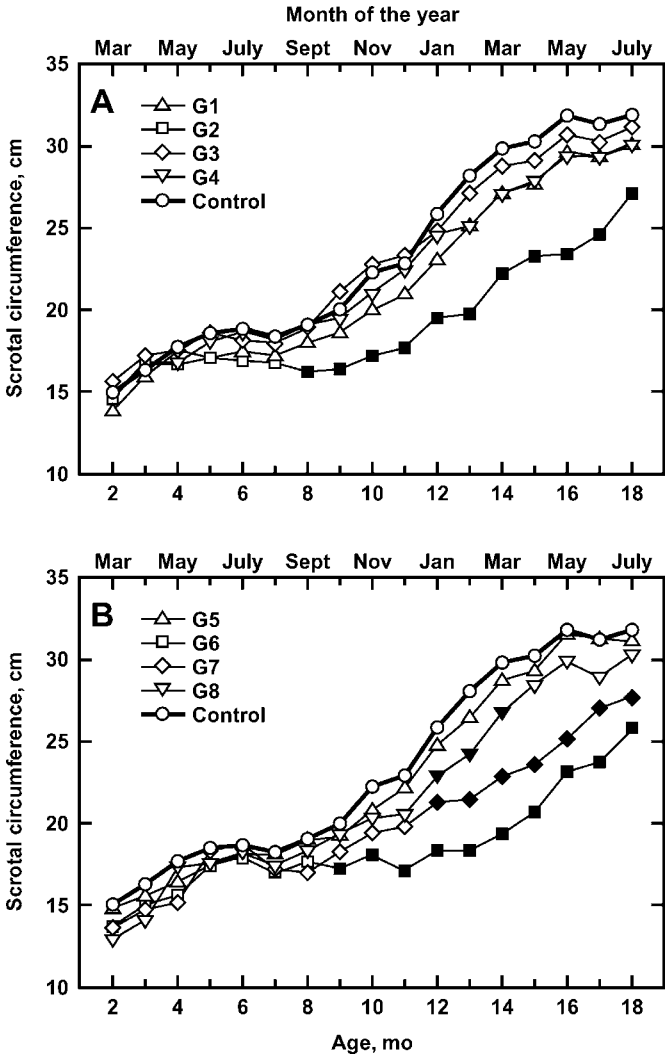

Figure 1. Scrotal circumference (SC; mean ± SE; n = 4 to 6 at each time point) in bull calves treated with the GnRH agonist, deslorelin, as follows: G1 = from birth to 3 mo of age; G2 = from 3 to 6 mo; G3 = from 6 to 9 mo; G4 = from 9 to 12 mo; G5 = from birth to 15 mo; G6 = from 3 to 15 mo; G7 = from 6 to 15 mo; G8 = from 12 to 15 mo; and untreated control group. Filled symbols indicate difference ($P < 0.05$) in SC from the control group at each specific time. Notice that the control group is depicted in both panels. Mar = March; Sept = September; Nov = November, Jan = January.

GATA-4 as a Marker for Bovine Sertoli Cells

The GATA-4 was abundant in the nucleus of every Sertoli cell (Figure 2). Within the seminiferous tubule,

Table 1. Least squares means (±SE) for characteristics at puberty of bulls treated with the gonadotropin-releasing hormone agonist, deslorelin, at different ages[a]

Group	Age at treatment, mo	No.	Bulls attaining puberty by 18 mo[b]	Age at puberty, d	BW at puberty, kg
G1	0 to 3	6	5/6	449 ± 17	316 ± 14
G2	3 to 6	5	2/5*	494 ± 27	345 ± 23
G3	6 to 9	6	6/6	444 ± 16	314 ± 13
G4	9 to 12	4	3/4	440 ± 22	303 ± 18
G5	0 to 15	6	6/6	459 ± 16	310 ± 13
G6[c]	3 to 15	6	1/6*	562	375
G7	6 to 15	5	4/5	506 ± 19*	333 ± 16
G8	12 to 15	6	5/6	454 ± 17	332 ± 14
G9	Control	6	6/6	420 ± 16	288 ± 13
Contrasts				*P*-value	
G1+G5 vs. control			11/12	0.09	0.147
G2+G6 vs. control			3/11*	0.001	0.005
G3+G7 vs. control			10/11	0.009	0.043

*Indicates different from control group, $P < 0.05$.
[a]Puberty was defined as the age when a bull reached 28 cm scrotal circumference. Mean comparisons were made against the control group for age and body weight at puberty (Dunnett's test for single treatments and contrasts for treatment combinations).
[b]Comparisons with the control group (Pearson χ^2 test).
[c]Only one bull attained puberty and no standard errors could be calculated for this group.

GATA-4 was specifically located in Sertoli cell nuclei and no positive staining was detected in germ cells, irrespective of their stage of development. Importantly, amount of GATA-4 was similar in Sertoli cell nuclei from testicular sections of both prepubertal (Figure 2A) and postpubertal (Figure 2B) bulls. The antibody used was specific for GATA-4, as no staining was observed in Sertoli cells when the GATA-4 procedure was used with nonimmune serum, in the absence of primary antibody, nor when the antibody was mixed with the GATA-4 blocking peptide (Figure 2C and D).

Testis Quantitative Morphometry and Daily Sperm Production

The percent distribution of tubular and interstitial tissue in the testes did not differ among groups (P = 0.46; Table 3), with an overall average of 70% of the space occupied by seminiferous tubules. The different stages of sexual development at the time of castration between some of the treated groups and the control group also were observed by microscopic assessment. The smallest tubule diameter was observed in G6 (P =

Table 2. Least squares means (±SE) for variables at castration (19 mo of age) of bulls treated with the gonadotropin-releasing hormone agonist, deslorelin, at different ages[a]

Group	Age at treatment, mo	No.	BW, kg	Scrotal circumference, cm	Paired testis weight, g	Paired parenchyma weight, g	Paired epididymis weight, g
G1	0 to 3	6	360 ± 11	30.0 ± 1.1	321 ± 41	298 ± 38	36.5 ± 3.8
G2	3 to 6	5	368 ± 13	27.1 ± 1.2*	217 ± 45*	202 ± 42*	30.6 ± 4.2
G3	6 to 9	6	377 ± 11	31.1 ± 1.1	369 ± 41	343 ± 38	42.2 ± 3.8
G4	9 to 12	4	381 ± 14	30.1 ± 1.4	348 ± 50	323 ± 47	41.5 ± 4.7
G5	0 to 15	5	363 ± 13	31.1 ± 1.2	380 ± 45	354 ± 42	41.8 ± 4.2
G6	3 to 15	6	364 ± 11	25.8 ± 1.1*	181 ± 41*	168 ± 38*	28.0 ± 3.8†
G7	6 to 15	5	346 ± 13	27.7 ± 1.2†	268 ± 45	249 ± 42	28.6 ± 4.2†
G8	12 to 15	4	369 ± 14	30.4 ± 1.4	376 ± 50	350 ± 47	38.5 ± 4.7
G9	Control	6	370 ± 11	31.8 ± 1.1	404 ± 41	375 ± 38	42.8 ± 3.8
Contrasts			*P*-value				
G1+G5 vs. control			0.547	0.365	0.306	0.306	0.445
G2+G6 vs. control			0.778	0.001	0.001	0.001	0.007
G3+G7 vs. control			0.558	0.089	0.104	0.104	0.127

†Indicates different from control group, $P < 0.10$.
*Indicates different from control group, $P < 0.05$.
[a]Mean comparisons were made with the control group (Dunnett's test for single treatments and contrasts for treatment combinations). Organ weights were on a fresh-tissue basis.

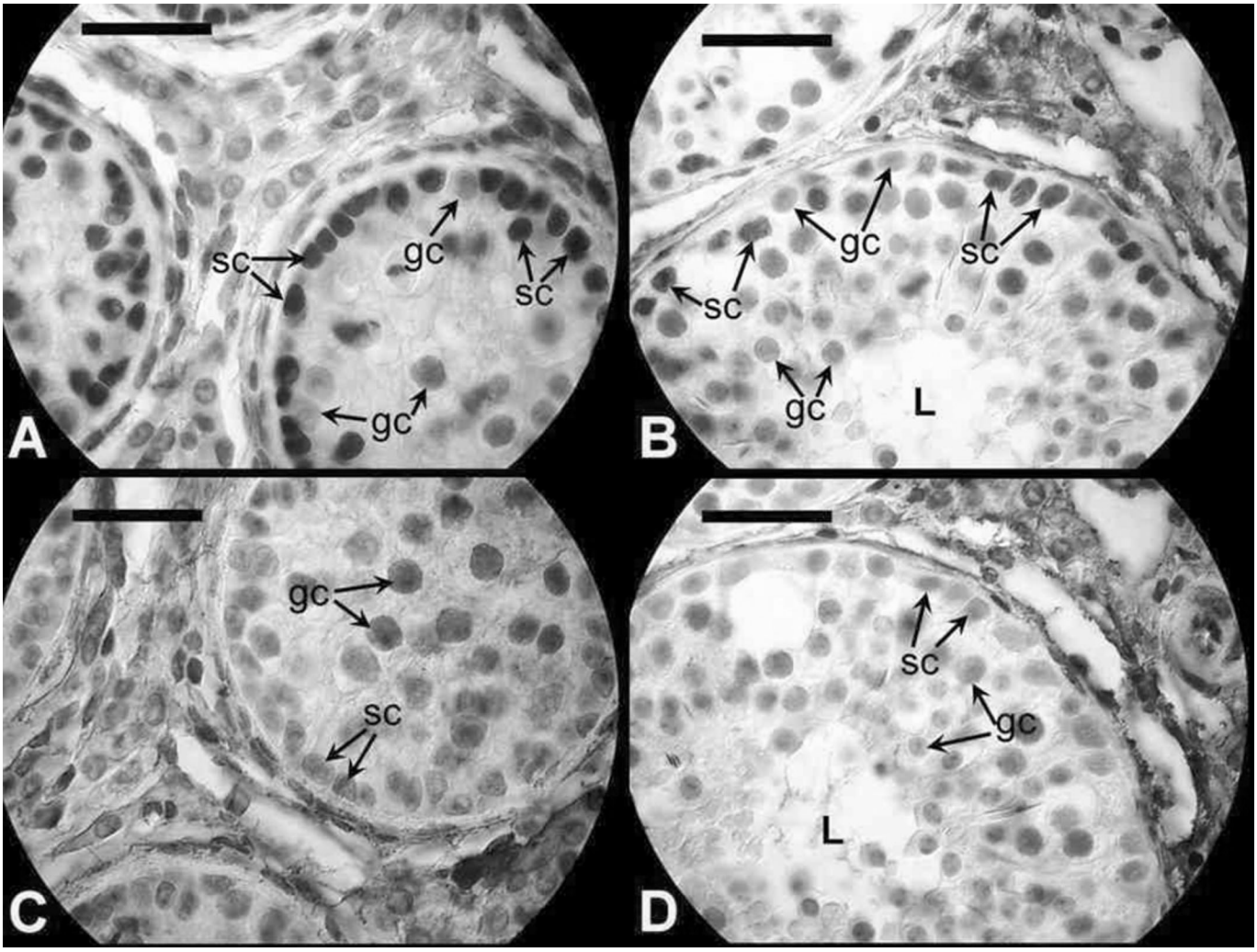

Figure 2. Immunolocalization of GATA-4 in Sertoli cell (sc) nuclei in a prepubertal (A) and postpubertal (B) Zebu bull. (Figure is available in color online at http://jas.fass.org/. Positive staining for GATA-4 is depicted by red staining.) Sections shown were counterstained with hematoxylin to illustrate that the GATA-4 procedure does not result in germ cell (gc) nuclei staining inside the seminiferous tubules. Panels C and D depict sections in which GATA-4 blocking peptide was used to confirm the specificity of the primary antibody in each bull, respectively. The appearance in Panels C and D was identical to sections stained only with hematoxylin, indicating the advantages of using the GATA-4 immunostaining procedures to identify Sertoli cell nuclei in bulls. The tubule lumen (L) is shown in the postpubertal bull in panels B and D. Magnification bar on each micrograph represents 50 μm.

0.07) and G2+G6 ($P = 0.06$). In addition, in G2, G6, and G7, less than 50% of seminiferous tubules had spermatids within the lumen compared with 82% in the control group. The total seminiferous epithelium volume in G2 and G6 was only half that observed in the control group ($P < 0.05$; Table 4), indicating a lesser capacity for sperm production. Consistent with this finding and with the decreased testis size in G2 and G6, DSP was affected by treatment ($P < 0.05$; Table 4). Bulls from G2, G6, and G7 produced less than 50% of the total sperm cells compared with the bulls of the control group; however, when DSP was expressed per gram of parenchymal tissue, the differences among treatments were not significant ($P = 0.59$). The Sertoli cell nuclear density area from the seminiferous epithelium and the Sertoli cell nuclear density volume from the total testis were greater in G6 ($P < 0.05$; Table 5) and G2+G6 ($P < 0.01$) than in the control group. In contrast, the mean Sertoli cell nuclear volume ($P = 0.29$) and total number of Sertoli cells were not affected by treatment ($P = 0.45$).

Some bulls in G2 and G6 had testis development comparable to control bulls and had attained puberty by 18 mo of age. Previous observations indicated that some animals can overcome the effects of treatment with GnRH agonist earlier (our unpublished observations). When data from the bulls reaching puberty in G2 and G6 were not included for statistical analyses, values for variables changed to the extent that treatment differences existed that were otherwise not detected (Table 6). For example, epididymis weight, tu-

Table 3. Least squares means (±SE) for seminiferous tubule characteristics in bulls treated with the gonadotropin-releasing hormone agonist, deslorelin, at different ages[a]

Group	Age at treatment, mo	No.	Tubular tissue, %	Average tubule diameter, μm	Short tubule diameter, μm	Tubules with spermatids, %
G1	0 to 3	6	71 ± 2	238 ± 11	202 ± 9	76 ± 9
G2	3 to 6	5	64 ± 3	203 ± 13	175 ± 10	39 ± 10*
G3	6 to 9	6	70 ± 2	226 ± 11	190 ± 9	74 ± 9
G4	9 to 12	4	70 ± 3	220 ± 14	186 ± 11	60 ± 11
G5	0 to 15	5	73 ± 3	230 ± 13	194 ± 10	79 ± 10
G6	3 to 15	6	71 ± 2	178 ± 11	151 ± 9†	29 ± 9*
G7	6 to 15	5	72 ± 3	213 ± 13	184 ± 10	46 ± 10†
G8	12 to 15	4	70 ± 3	226 ± 14	188 ± 11	79 ± 11
G9	Control	6	68 ± 2	216 ± 11	184 ± 9	82 ± 9
Contrasts			*P*-value			
G1+G5 vs. control			0.198	0.207	0.219	0.704
G2+G6 vs. control			0.793	0.085	0.064	0.001
G3+G7 vs. control			0.405	0.785	0.797	0.07

†Indicates different from control group, $P < 0.10$.
*Indicates different from control group, $P < 0.05$.
[a]Mean comparisons were made with the control group (Dunnett's test for single treatments and contrasts for treatment combinations).

bule diameters, and Sertoli nucleus volume were smaller in G2 and G6 than in the control group. In addition, differences between G2 and G6 and the control group for other variables, such as SC, paired testis, and parenchymal weights, and DSP became more evident when data were analyzed in this manner.

Serum Luteinizing Hormone and Testosterone Concentrations

Serum LH concentrations are depicted in Figure 3. Only the effect of age was significant ($P < 0.001$), with no effect of treatment or the interaction of treatment × age. The overall profile of LH for all groups showed a bimodal tendency, with the greatest concentrations detected around 7 mo of age (August) and the least concentrations around 3 (April) and 13 mo (February) of age.

Serum T concentrations are depicted in Figure 4. The effects of treatment ($P < 0.05$), age ($P < 0.001$) and treatment × age interaction ($P < 0.001$) were significant. In bulls of the control group, T concentrations remained less than 0.5 ng/mL, which was not different from zero ($P = 0.20$ to 0.91), until 9 mo of age. From 10 mo onward,

Table 4. Least squares means (±SE) for seminiferous epithelium characteristics and daily sperm production (DSP) of bulls treated with the gonadotropin-releasing hormone agonist, deslorelin, at different ages[a]

Group	Age at treatment, mo	No.	Seminiferous epithelium volume: Percentage of testes	Seminiferous epithelium volume: Total per paired testes, cm^3	DSP, millions/g of parenchyma	DSP, millions/ paired testes
G1	0 to 3	6	60 ± 2.6	171.9 ± 24.2	19.16 ± 3.03	5,923.97 ± 1105
G2	3 to 6	5	56 ± 2.8	108.7 ± 26.6*	17.36 ± 3.32	3,830.15 ± 1210†
G3	6 to 9	6	60 ± 2.6	195.5 ± 24.2	22.63 ± 3.03	7,493.07 ± 1105
G4	9 to 12	4	59 ± 3.2	191.9 ± 29.7	22.74 ± 3.71	7,090.49 ± 1353
G5	0 to 15	5	62 ± 2.8	211.1 ± 26.6	21.61 ± 3.32	7,733.45 ± 1210
G6	3 to 15	6	60 ± 2.6	95.8 ± 24.2*	17.70 ± 3.03	3,369.10 ± 1105*
G7	6 to 15	5	62 ± 2.8	149.3 ± 26.6	15.26 ± 3.32	3,912.02 ± 1210†
G8	12 to15	4	60 ± 3.2	196.7 ± 29.7	23.93 ± 3.71	8,457.59 ± 1353
G9	Control	6	59 ± 2.6	209.2 ± 24.2	22.38 ± 3.03	8210.16 ± 1105
Contrasts			*P*-value			
G1+G5 vs. control			0.401	0.562	0.60	0.322
G2+G6 vs. control			0.896	0.001	0.206	0.002
G3+G7 vs. control			0.428	0.231	0.369	0.076

†Indicates different from control group, $P < 0.10$.
*Indicates different from control group, $P < 0.05$.
[a]Mean comparisons were made with the control group (Dunnett's test for single treatments and contrasts for treatment combinations).

Table 5. Least squares means (±SE) for Sertoli cell characteristics of bulls treated with the gonadotropin-releasing hormone agonist, deslorelin, at different ages[a]

Group	Age at treatment, mo	No.	Sertoli cell nuclei: Percentage area of seminiferous epithelium	Sertoli cell nuclei: Percentage volume of total testis	Sertoli cell nuclei: Mean volume, μm³	Sertoli cell number, billions/paired testes
G1	0 to 3	6	8.8 ± 1.8	5.3 ± 1.0	315 ± 28	13.91 ± 1.38
G2	3 to 6	5	15.8 ± 1.9†	8.5 ± 1.1	272 ± 30	16.80 ± 1.51
G3	6 to 9	6	9.6 ± 1.8	5.6 ± 1.0	341 ± 28	14.92 ± 1.38
G4)	9 to 12	4	11.0 ± 2.2	6.1 ± 1.2	314 ± 34	16.36 ± 1.69
G5	0 to 15	5	8.7 ± 1.9	5.3 ± 1.1	350 ± 30	15.52 ± 1.51
G6	3 to 15	6	16.3 ± 1.8*	10.0 ± 1.0*	248 ± 28	16.16 ± 1.38
G7	6 to 15	5	13.3 ± 1.9	8.2 ± 1.1	321 ± 30	16.28 ± 1.51
G8	12 to 15	4	10.0 ± 2.2	5.9 ± 1.2	327 ± 34	19.05 ± 1.69
G9	Control	6	9.1 ± 1.8	5.3 ± 1.0	307 ± 28	17.66 ± 1.38
Contrasts			*P*-value			
G1+G5 vs. control			0.856	0.991	0.464	0.095
G2+G6 vs. control			0.003	0.004	0.179	0.497
G3+G7 vs. control			0.304	0.203	0.486	0.238

†Indicates different from control group, $P < 0.10$.
*Indicates different from control group, $P < 0.05$.
[a]Mean comparisons were made with the control group (Dunnett's test for single treatments and contrasts for treatment combinations).

T concentrations increased and varied between 0.8 and 4 ng/mL. No clear differences in T concentration were detected between bulls of the treated and control groups during the treatment period. Concentrations of T were greater in bulls from some of the treated groups than in the control group at specific times when comparisons were made: G1 at 10 mo of age ($P < 0.05$); G2 at 13 mo ($P = 0.10$); G3 from 9 to 12 mo ($P < 0.05$); G5 at 6 and 7 mo ($P < 0.05$); G6 at 7 mo ($P = 0.10$); G7 around 9 mo ($P < 0.05$); and G8 at 9 and 10 ($P = 0.10$) and 15 to 17 mo of age ($P < 0.05$). Earlier increases in T concentrations ($P < 0.05$) were detected in G1, G5, G6, G7, and G8 by 6 to 8 mo of age compared with the pattern of T increase in the control group.

Discussion

Treatment with GnRH agonist had a differential effect on testis development, depending on duration and age at the beginning of treatment. The most remarkable effect was observed in G2 and G6 (treatment in both groups started at 3 mo of age). Only 3 out of 11 bulls in these groups attained puberty (28 cm SC) by 18 mo of

Table 6. Least squares means (±SE) for testicular variables at castration, daily sperm production (DSP) and quantitative morphometry (fresh-tissue basis) of bulls treated with the gonadotropin-releasing hormone agonist, deslorelin, at different ages, when data from bulls attaining puberty in G2 (treatment from 3 to 6 mo of age) and G6 (treatment from 3 to 15 mo of age) groups were excluded from the analyses[a]

Variable	G2	G6	Control	G2+G6 vs. control
No.	3	5	6	
				P-value
Scrotal circumference, cm	25.0 ± 1.5*	25.3 ± 1.2*	31.8 ± 1.1	0.001
Paired testis weight, g	138 ± 56*	171 ± 43*	404 ± 41	0.001
Paired epididymis weight, g	23.7 ± 5.2*	27.6 ± 4.0*	42.8 ± 3.8	0.001
Paired parenchyma weight, g	129 ± 52*	159 ± 40*	375 ± 38	0.001
DSP, millions/paired testes	1691.4 ± 1504*	3505.4 ± 1165*	8210.2 ± 1105	0.001
Tubules with spermatids, %	12 ± 11***	27 ± 9***	82 ± 9	0.001
Average tubule diameter, μm	171 ± 15	176 ± 11	216 ± 11	0.004
Short tubule diameter, μm	153 ± 12	151 ± 9†	184 ± 9	0.006
Sertoli nucleus volume, μm³	218 ± 38	246 ± 29	307 ± 28	0.042

†Indicates different from control group, $P < 0.10$.
*Indicates different from control group, $P < 0.05$.
***Indicates different from control group, $P < 0.001$.
[a]Mean comparisons were made with the control group (Dunnett's test for single treatments and contrasts for treatment combination).

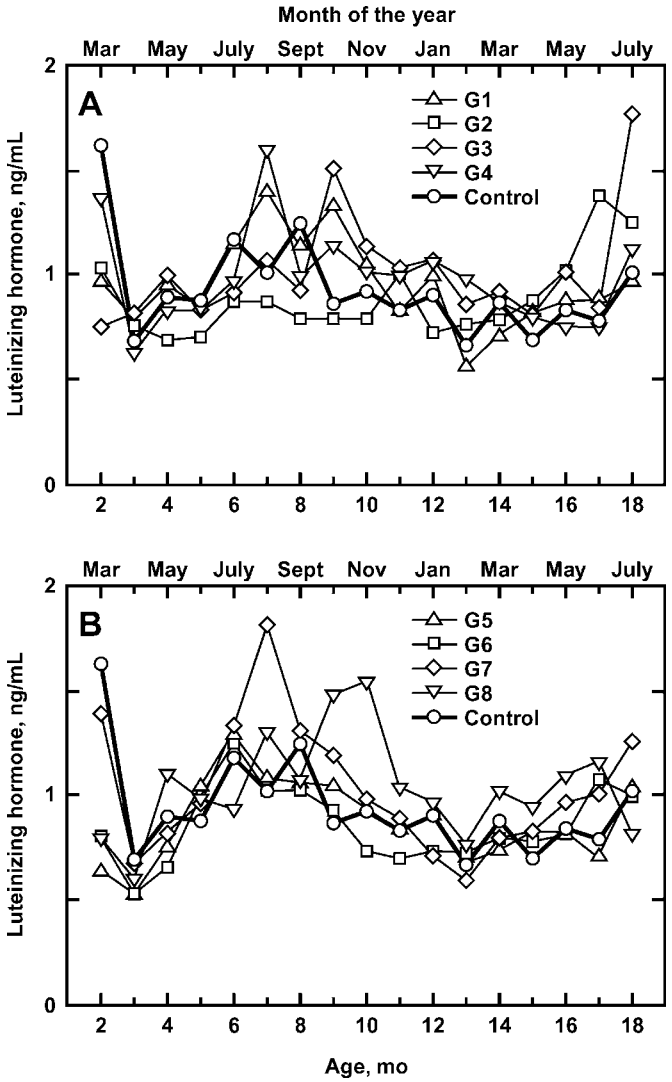

Figure 3. Luteinizing hormone concentrations (mean ± SE; n = 4 to 6 at each time point) in bull calves treated with the GnRH agonist, deslorelin, as follows: G1 = from birth to 3 mo of age; G2 = from 3 to 6 mo; G3 = from 6 to 9 mo; G4 = from 9 to 12 mo; G5 = from birth to 15 mo; G6 = from 3 to 15 mo; G7 = from 6 to 15 mo; G8 = from 12 to 15 mo; and the untreated control group. Filled symbols indicate differences ($P < 0.05$) in LH concentration from the control group at each specific time. Notice that control group is depicted in both panels. Mar = March; Sept = September; Nov = November, Jan = January.

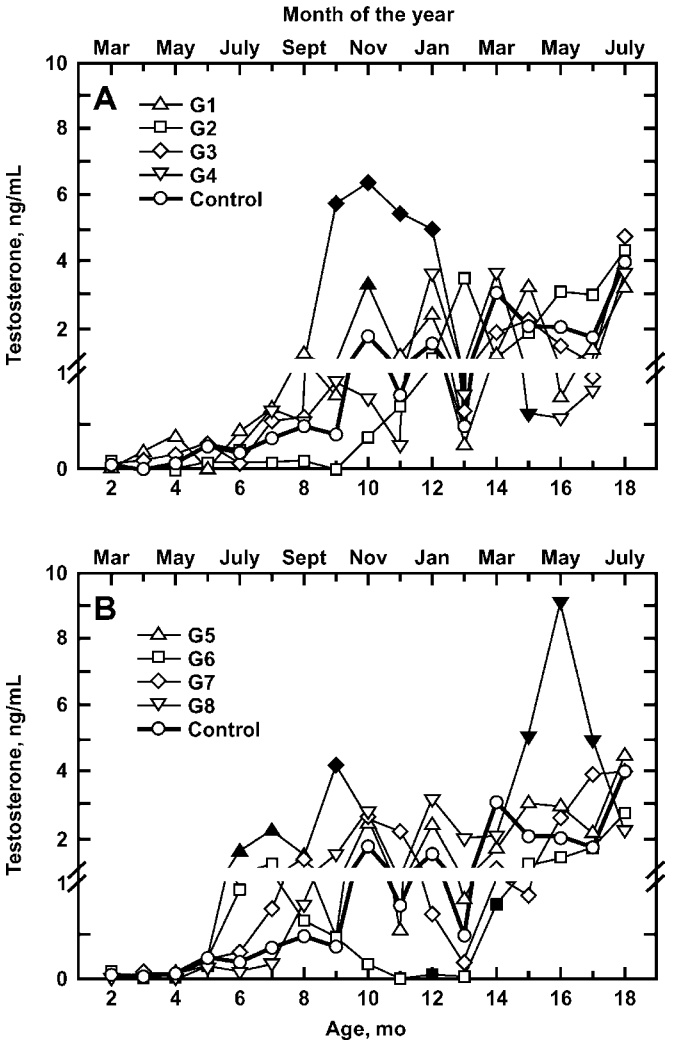

Figure 4. Testosterone concentrations (T; mean ± SE; n = 4 to 6 at each time point) in bull calves treated with the GnRH agonist, deslorelin, as follows: G1 = from birth to 3 mo of age; G2 = from 3 to 6 mo; G3 = from 6 to 9 mo; G4 = from 9 to 12 mo; G5 = from birth to 15 mo; G6 = from 3 to 15 mo; G7 = from 6 to 15 mo; G8 = from 12 to 15 mo; and the untreated control group. Filled symbols indicate difference ($P < 0.05$) in T concentration from the control group at each specific time. Notice that control group is depicted in both panels. Mar = March; Sept = September; Nov = November, Jan = January.

age, and the bulls of these groups that attained puberty were older at puberty than those of the control group.

The importance of Sertoli cell number and function on spermatogenesis and sperm production is well known (Sharpe et al., 2003). In the present study, there is an apparent discrepancy between the decrease in seminiferous epithelium and sperm production in G2 and G6 and the lack of an effect of treatment on the number and size of Sertoli cells. It is possible that the heterogeneous response to deslorelin within treatment groups accounted for part of this lack of effect. For this reason, when data of bulls reaching puberty in G2 and G6 groups were not included in the analyses, some negative effects were observed in tubule diameter and Sertoli nuclear volume in these groups. Even though Sertoli cell number was not affected by any of the treatments, the subtle decrease in nuclear volume in G2 and G6 indicates a possible decrease in Sertoli cell function, which might help explain the impaired spermatogenesis and decreased sperm production in these bulls.

Continuous treatment with GnRH agonists in prepubertal and young postpubertal bulls increases mean T

(D'Occhio and Aspden, 1996) and basal LH (Jiménez-Severiano et al., 2003) concentration associated with an increase in testis size (D'Occhio and Aspden, 1996). In contrast, when this treatment is administered to very young bulls (6 to 18 wk of age), there is a decrease in gonadotropin and T concentrations (Chandolia et al., 1997). In both cases, episodic LH release is suppressed. It is possible that a critical period for testis development might have occurred around 3 to 6 mo of age that was disrupted by treatment with GnRH agonist. In young bulls undergoing typical sexual development, there is a transient elevation of LH concentration between 6 and 20 wk of age, resulting from increased pulse frequency and amplitude (Amann et al., 1983; Evans et al., 1993; 1995), which precedes cell differentiation and testis growth (Amman, 1983; Evans et al., 1996). The negative effects of continuous treatment with GnRH agonist at early ages might result from inhibition or delay of this LH increase, resulting in decreased testis growth and decreased numbers of spermatocytes and spermatids (Chandolia et al., 1997), which agrees with the decreased sperm production in G2 and G6 groups in the present study.

Intuitively, a similar response to that detected in G2 and G6 would be anticipated in bulls of G5 (treatment from birth to 15 mo) because the effect of constant infusion with deslorelin on the hormonal milieu also should be present from 3 to 6 mo of age in bulls of the G5 group. This would, however, assume that the implants continued to release deslorelin for greater than 3 mo. Although this might be expected based on the formulation of the implant (D'Occhio and Aspden, 1996), in previous research we have observed heifers reinitiating estrous cycles after 3 mo of treatment with similar implants (our unpublished observations). It cannot be assumed, therefore, that bulls in the G5 group were necessarily under the influence of deslorelin at 3 mo of age. It is unlikely, however, that all six bulls in G5 had overcome the effects of deslorelin at or before 3 mo of age.

A second possible explanation for the inhibitory effects on testis development observed in the G2 and G6 groups is the initial effect on hormone concentration, caused by agonist infusion, which is characterized by acute increases of LH, FSH, and T (D'Occhio and Aspden, 1996; Jiménez-Severiano et al., 2003) that may last up to 24 h before returning to basal concentrations (Jiménez-Severiano, 2001), with a second increase in both LH, which lasts for 2 to 7 d, and T, which remains greater for the entire treatment period (D'Occhio and Aspden, 1996; Jiménez-Severiano et al., 2003). Therefore, even though bulls in the G5 were under the chronic effect of treatment by 3 mo of age, they did not experience the acute hormone increase at that age, as did bulls in G2 and G6.

Treatment with T or other androgens to animal models in which spermatogenesis has been disrupted by different means (Meistrich et al., 1999; Setchell et al., 2001; Shetty et al., 2001) results in greater intratesticular T concentration, which inhibits the restoration of spermatogonial number and differentiation, and normal resumption of spermatogenesis (Meistrich et al., 1999; Setchell et al., 2001; Shetty et al., 2002). In these models, spermatogonia are the only germ cell present in the seminiferous tubules (Kangasniemi et al., 1996; Meistrich et al., 1999; Tohda et al., 2001), which is comparable to what is observed in bulls younger than 20 wk of age (Evans et al., 1996).

Greater intratesticular T concentration might inhibit spermatogonial differentiation by increasing amounts of apoptotic factors or by repressing amounts of differentiation or surviving factors (Shetty et al., 2001). Some of these proteins, such as stem cell factor and glial cell line-derived neurotrophic factor, are normally secreted by Sertoli cells and are important in regulating spermatogonial proliferation and differentiation (Blanchard et al., 1998; Meng et al., 2000). The negative effects of T in these experimental models seem to be mediated by direct action on androgen receptors in the gonad (Shetty et al., 2000; Tohda et al., 2001). Because Sertoli cells have receptors for androgens and spermatogonia do not, any possible action on spermatogenesis should be mediated by receptors on Sertoli cells (Zhu et al., 2000; De Rooij, 2001). A possible effect of T on Sertoli cell function agrees with our findings of smaller Sertoli cell nuclear volume, which might indicate a decreased function of these cells.

Testosterone is not the most important androgen produced by the testes of bulls at 3 mo of age (Amman, 1983; Moura and Erickson, 1997); however, other androgens, such as androstenedione, might induce similar effects on Sertoli cells at this earlier age (Shetty et al., 2002). Certainly we did not detect any significant increase in LH or T concentrations, temporally associated with the beginning of treatment. Given the episodic nature of LH and T secretion, however, it is possible that our schedule of sampling (only one sample per month) and the small number of animals per group did not allow for detection of possible changes in hormone concentration.

Another important finding was that GATA-4 specifically stained Sertoli cell nuclei within the seminiferous tubule in pre- and postpubertal bulls, with no germ cells being positive to GATA-4. Sertoli nuclear staining was very intense, and the boundaries of each nucleus were clearly delineated. The GATA-4 staining enabled rapid identification of Sertoli nuclei using light microscopy and greatly increased the accuracy and speed of identification and enumeration procedures in both sexually mature and immature bulls. Based on the consistent and specific staining of the Sertoli nucleus inside the seminiferous tubule, we conclude that GATA-4 is a reliable marker for Sertoli cells in pre- and postpubertal bulls.

There is some inconsistency in findings regarding the age at which GATA-4 protein is down-regulated during sexual development. The staining of GATA-4 in Sertoli nuclei in relatively old bulls (even though some of them

were still prepubertal) agrees with data reported in pigs (McCoard et al., 2001), in which Sertoli cells contained GATA-4 throughout fetal, postnatal, and adult life. In contrast, GATA-4 apparently is downregulated in mouse Sertoli cells after 14 d of age, in such a way that Sertoli cells from pubertal or adult mice no longer contain GATA-4 protein (Viger et al., 1998). Although this might be due to intrinsic species differences, chances are that variations in the sensitivity of the staining procedures could account for some of the differences observed among species. It has been recently reported that GATA-4 protein is present throughout postnatal development in mouse Sertoli cells, using different in vivo and in vitro systems (Imai et al., 2004), suggesting that GATA-4 has an important role in regulating Sertoli cell function not only during sexual development, but also during adult life.

In conclusion, treatment with deslorelin had no apparent beneficial effect on testis development and delayed puberty when treatment was initiated at 3 mo of age. The mechanisms involved in sexual retardation might include suppression of the maturational increase in LH, negative effects on testis development of the acute initial increase after treatment of gonadotropin and testosterone, or both. We also have shown for the first time that staining for GATA-4 is a useful method for identifying and quantifying Sertoli cell nuclei in both pre- and postpubertal bulls.

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